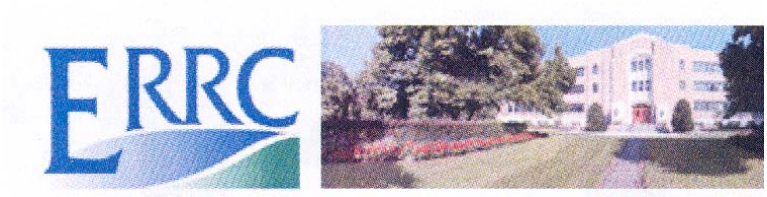

EASTERN REGIONAL RESEARCH CENTER
AGRICULTURAL RESEARCH SERVICE
UNITED STATES DEPARTMENT OF AGRICULTURE
600 E. MERMAID LANE
WYNDMOOR, PA 19038
(215) 233-6400

Title: Growth Kinetics of *Clostridium perfringens* in Cooked Beef

Author(s): L. Huang

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GROWTH KINETICS OF *CLOSTRIDIUM PERFRINGENS* IN COOKED BEEF[1]

LIHAN HUANG[2]

Food Safety Intervention Technologies Research Unit
Eastern Regional Research Center
USDA Agricultural Research Service
600 East Mermaid Lane
Wyndmoor, PA 19038

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ABSTRACT

The objective of this work was to investigate the growth kinetics of a three-strain cocktail of Clostridium perfringens *in cooked beef. The study was conducted by growing the heat-activated spores in ground beef under isothermal conditions between 17-50C. A four-parameter Gompertz equation was used as a primary model to fit the growth curves along with a modified Ratkowsky model to analyze the temperature dependence of the bacterial growth. Results indicated that the Gompertz model could accurately describe the growth of* C. perfringens *in cooked beef. The estimated theoretical minimum, optimum, and maximum growth temperatures of this organism in cooked beef were 9.8, 47.1, and 50.8C, respectively. A linear relationship between the durations of the lag and exponential phases of growth curves was observed in this study. Such a linear relationship can be used to generate a linear isothermal growth curve complete with the lag, exponential, and stationary phases without complex mathematical computation. The kinetic models and growth parameters obtained from this study potentially can be applied to the food industry to design appropriate cooling schedules and estimate the growth of* C. perfringens *in thermally processed beef products under temperature abuse conditions.*

[1] Mention of a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

[2] Corresponding author. USDA ARS Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA 19038. TEL: (215)233-6552; FAX : (215)233-6406; Email: lhuang@arserrc.gov

INTRODUCTION

Clostridium perfringens is an anaerobic, Gram-positive, spore-forming bacillus ubiquitously distributed in the environment. This organism can be readily isolated from dust, raw meat and poultry, and the intestinal tract of man and animals (Granum 1990). Symptoms of *C. perfringens* gastroenteritis include acute abdominal pain and diarrhea. These symptoms usually appear 8-16 h after ingestion of the bacteria and can last 24-48 h (CDC 1996; Hall and Angelotti 1965). *C. perfringens* is a common infectious cause of outbreaks of foodborne illness in the United States, and is a major concern to the food industry (Stringer *et al.* 1980; CDC 1994, 1996; Todd *et al.* 1997).

Food poisoning caused by this organism is primarily attributed to the consumption of foods contaminated with large numbers of vegetative cells capable of producing enterotoxin in the intestine (Duncan *et al.* 1972). Cooked meat and poultry products are frequently associated with foodborne *C. perfringens* outbreaks (Collee *et al.* 1961; Hall and Angelotti 1965; Shandera *et al.* 1983; Gross *et al.* 1985; Bean *et al.* 1990). Due to high heat resistance of the spores, they can survive normal cooking conditions for meat products and are thermally activated following the heating (Duncan *et al.* 1972). Because heating also causes the depletion of oxygen and subsequently creates an anaerobic condition in cooked products, the heat-activated spores may germinate, outgrow, and multiply to a dangerously high dose level if suitable temperatures are maintained (Gross *et al.* 1985). According to Barnes *et al.* (1963), almost all *C. perfringens* spores germinated in beef after heating, while only 3% of germination was observed in raw beef. Because of the wide distribution of this organism in the environment, the presence of *C. perfringens* in the final products is almost unavoidable (Rhodehamel and Harmon 2001).

Outbreaks of *C. perfringens* food poisoning are most likely caused by improper food handling practices after cooking (Craven 1980; Juneja *et al. 1994*). This organism can grow in a wide temperature range between 10-52C (Hall and Angelotti 1965; Johnson 1990; FDA 1998) with very short generation times (7-30 min) between 30-47C. Maintaining cooked meat products within this temperature range for extended time periods will result in a rapid growth of this organism.

Rapid cooling after thermal processing is essential to prevent the outgrowth of *C. perfringens* spores in cooked meat and poultry products. The U.S. Department of Agriculture (USDA) recommends that the internal temperature of the slowest cooling point in cooked beef, roast beef, and cooked corned beef be cooled from 48.9C to 12.8C in 6 h or less, and the cooling should continue to 4.4C prior to boxing (USDA 1993). The time/temperature guidelines, issued by the Food Safety Inspection Service (FSIS) of USDA in a directive (USDA-FSIS 1989), require the maximum internal temperature should not be maintained between 54.4C and 26.7C for more than 1.5 h nor between 26.7C and 4.4C for more than 5 h. The Food Code

ublished by the U.S. Food and Drug Administration (FDA) also suggests that all ood should be cooled from 60C to 21C in 2 h and from 21C to 5C in 4 h (FDA 001).

Despite the importance of *C. perfringens* as a potential foodborne hazard, few tudies have been conducted to systematically understand the growth kinetics of this athogen in cooked beef. Blankenship *et al.* (1988) developed a model for redicting the outgrowth of *C. perfringens* spores in cooked chili. Labbe and Huang (1995) investigated the growth behavior of *C. perfringens* in laboratory nedia and ground beef, but the temperature of the study was limited to the optimum ange (37-48C). Juneja and Majka (1995), on the other hand, studied the outgrowth f *C. perfringens* spores in cook-in-bag beef products at sub-optimal temperatures 15 and 28C). Other studies have been conducted to understand the growth of *C. erfringens* in meat products under dynamic conditions (Juneja *et al.* 1994; Taormina *et al.* 2003). These studies do not provide enough information on the rowth kinetics of this organism. Juneja *et al.* (1999) conducted a study to nderstand the growth kinetics of *C. perfringens* and developed a predictive model or growth of this organism in tryticase-peptone-glucose-yeast (TPGY) extract roth. Since the growth kinetics of *C. perfringens* was collected in the broth ystem, the results may not be accurate when applied to real meat products.

The main objective of this study was to understand the growth kinetics of *C. perfringens* in cooked beef under isothermal conditions and develop nathematical models to describe the bacterial growth. The kinetic models leveloped from this study could be potentially applied to cooked beef products in he food industry to design appropriate cooling processes that would prevent the utgrowth of this pathogen and estimate the growth of *C. perfringens* during the listribution and storage of cooked beef products under temperature abuse onditions.

MATERIALS AND METHODS

Test Organisms and Sample Inoculation

Three strains of *C. perfringens*, NCTC 8238, NCTC 8239, and ATCC 10388 were selected in this study. The bacterial strains were identical to those used by Juneja *et al.* (1999) and were obtained from Dr. John Novak of USDA-ARS-ERRC. Spore crops of these strains were grown and harvested using procedures developed by Juneja *et al.* (1993). Each spore crop was washed twice, resuspended in sterile distilled water, and maintained at 4C until use.

Ground beef (93% lean) was purchased from a local grocery store and sterilized by ionizing γ irradiation to a dose level of 42 KGy at –30C using a Cs^{137} (Thayer *et al.* 1995). The bacterial spores with the same optical density were mixed to form a 10 mL cocktail and then inoculated to approximately 1500 g of ground beef. The bacteria and ground beef mixture was thoroughly mixed in a Kitchen-Aid mixer (Model Max Watts 325), and a homogeneous distribution of bacterial cells was experimentally confirmed. The concentration of *C. perfringens* spores inoculated ground beef was approximately 100 spores per gram of meat.

The inoculated ground beef was divided into 5 ± 0.02 g portions and packaged into sterile plastic filter bags (12 × 19 cm, Model BagPage® BP 100, Topac Inc., Hingham, MA) and sealed under a final pressure level of 15 mmHg. Samples were kept frozen (-20C) until used in the experiment (< 1 month).

Growth Study

Frozen beef samples containing *C. perfringens* spores were thawed overnight in a refrigerator (4C). Prior to the experiment, ground beef samples were heat-shocked at 75C for 20 min in a water bath to activate the spores and inactivate any contaminating vegetative cells. The heat-shocked samples were then briefly rinsed with running water (≈20C for 1-2 min) and placed into incubators or water baths maintained at 17, 25, 30, 36, 45, 47, and 50 ± 0.5C, respectively. Samples from each incubation temperature were periodically removed for determination of bacterial cell concentrations.

Determination of Bacterial Cell Concentration

Samples removed from incubators/water baths were immediately diluted with equal volumes (5 mL) of 0.1% sterile peptone water. A rubber hammer was used to gently break and tenderize the meat samples in the plastic bags. The samples were then mixed in a MiniMix Stomacher (Model BagMixer® 100 W, Interscience Co., France) at the maximum speed for 12 min to completely homogenize the meat. After homogenization, a small volume (0.1-0.5 mL) of the liquid fraction was serially diluted with 0.1% sterile peptone-water and surface-plated onto Shahidi-Ferguson perfringens (SFP) agar. After that, each plate was overlaid with approximately 10 mL of freshly prepared SFP agar. Upon the solidification of the agar overlay, the plates were placed in an anaerobic chamber (Model Bactron IV, Sheldon Manufacturing Inc., Cornelius, OR) and incubated at 37C for 24-48 h under an atmosphere of $CO_2/N_2/H_2$ (10%:85%:5%). Growth experiments for each temperature were replicated in triplicate to generate isothermal growth curves.

Analysis of Growth Curves

A four-parameter Gompertz model (Eq. 1) used by Gibson *et al.* (1987) was

selected in this study as a primary model to describe the growth of *C. perfringens* in cooked beef under isothermal conditions. Plate counts of *C. perfringens* were transformed to log values and then fitted to Eq. (1) by nonlinear regression.

$$L(t) = A + (B-A)\exp\{-\exp[-\mu(t-M)]\} \qquad (1)$$

In Eq. (1), L(t) is the $\log_{10}$ count of the number of *C. perfringens* at time t, A is the initial cell concentration or the asymptotic $\log_{10}$ count as time approaches to zero in the models, and B is the maximum cell concentration or the asymptotic $\log_{10}$ count as time increases to infinity. The parameter μ is the relative growth rate at time M point, and M is the time at which the absolute growth rate is the maximum.

The experimental growth data were fitted to the Gompertz model using NCSS 2000 a Windows based statistical package (Hintze 1999). The parameters in the Gompertz model, A, B, μ, and M, were determined using a nonlinear regression procedure provided in NCSS 2000. Since nonlinear regression was used to obtain the nonlinear Gompertz model, a pseudo-R^2 value was constructed in NCSS 2000 for each growth curve to approximate the usual R^2 values used in linear regression. Although not perfect for evaluating the goodness of fit of the nonlinear growth models, the pseudo-R^2 values served well for comparative purposes.

Lag phase Duration and Specific Growth Rate

The Gompertz model was used to derive the secondary growth parameters, such as specific growth rate (K) and lag phase duration (λ) of a bacterial growth process under an isothermal condition (Eq. 2-3).

$$K = \frac{(B - A)\mu}{2.718} \qquad (2)$$

$$\lambda = M - \frac{1}{B - A} \qquad (3)$$

Effect of Temperature on Growth Parameters

A modified Ratkowsky model (Eq. 4) used by Zwietering *et al.*(1991) was chosen as a secondary model for describing the effect of temperature on growth parameters such as specific growth rate (K) and lag phase duration (λ). In Eq. (4), the dependent variable ϕ may be either K or $1/\lambda$. T_{min} and T_{max} are the theoretical minimum and maximum growth temperatures defined by this empirical model. During nonlinear regression, the minimum and maximum growth temperatures (T_{min} and T_{max}) were first determined from Eq. (4) using K and T and then directly used to estimate the coefficients (a and b) for $1/\lambda$. Since the minimum, optimum, and

maximum growth temperatures for *C. perfringens* reported in the literature are around 10-15C, 43-47C, and 50-52C (Labbe 1989; Wrigley 1994; FDA 1998), respectively, the optimum temperature is very close to the maximum growth temperature. The growth rate drops dramatically at temperature above the optimum temperature. Therefore, more experiments were conducted at temperatures below the optimum temperature (45-47C). Due to the unique shape of the ϕ-T curves, it is possible to obtain the coefficients in Eq. (4) with limited temperature data points.

$$\phi = a(T - T_{min})^2 \{1-\exp[b(T-T_{max})]\} \quad (4)$$

RESULTS AND DISCUSSION

Growth of *C. perfringens* Under Isothermal Conditions

The initial inoculum experimentally detected in this study was between 1.87–2.10 $\log_{10}$(CFU/g) with a maximum standard error of 0.07, indicating that the spores of *C. perfringens* were homogeneously mixed in the samples. When the samples were incubated under isothermal conditions, one would have expected that the cell population remain at the initial level during the lag phase. For *C. perfringens*, however, a slight decline in cell counts was observed before the numbers of bacterial counts started to increase. The decline in the cell concentration during the initial incubation stage was more severe at temperatures above 45C. This observation was probably related to a complex "Phoenix phenomenon" previously reported by Collee *et al.* (1961) and Shoemaker and Pierson (1976). The maximum decline in log cell counts during the initial period observed in this study was approximately 20% or 0.4 $\log_{10}$(CFU/g). If the Gompertz model was directly used to fit the raw growth data, the initial decline in the cell concentration would cause the model to converge to a lower value of A for a growth curve. So for practical purposes in this study, the Phoenix phenomenon was ignored and the raw data were adjusted to the initial inoculum level during nonlinear regression.

After the adjustment, all the growth data could be fitted to the Gompertz model with pseudo-R^2 values very close to 1.0 (Fig. 1). The maximum growth temperature was experimentally validated since no growth was observed at 51C. The average initial cell concentration, as represented by A, obtained from the Gompertz equations, was 1.90 ± 0.02 $\log_{10}$(CFU/g). This value agrees very closely with the experimentally determined initial concentrations from the samples. For all the beef samples, the average maximum cell concentration determined experimentally was 8.27 ± 0.06 $\log_{10}$(CFU/g), indicating that the maximum growth potential for *C. perfringens* in cooked beef was approximately 6.4 $\log_{10}$(CFU/g) in this study. The average maximum cell concentration was accurately estimated from the Gompertz models (8.26 ± 0.07 $\log_{10}$(CFU/g)). The closeness of A and B values to the

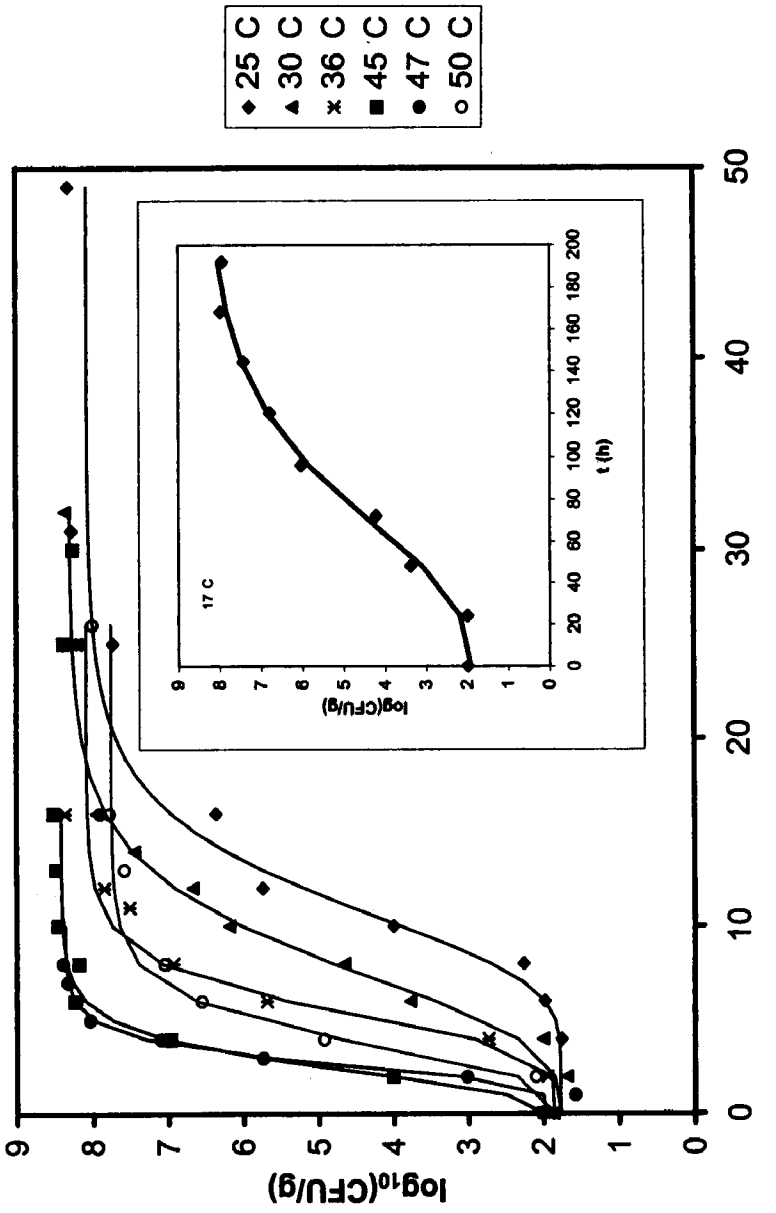

FIG. 1. REPRESENTATIVE GROWTH CURVES OF *C. PERFRINGENS* IN COOKED BEEF UNDER ISOTHERMAL CONDITIONS

TABLE 1.
ESTIMATED PARAMETERS OF MODIFIED RATKOWSKY MODEL FOR SPECIFIC GROWTH RATE (K) AND LAG PHASE DURATION (λ)

Parameters	Estimated value	Standard error
a_k^*	2.01×10^{-3}	5.94×10^{-4}
b_k^*	0.703	0.529
a_λ^{**}	4.60×10^{-4}	1.95×10^{-5}
b_λ^{**}	1.29	0.16
T_{min}	9.8	3.5
T_{max}	50.8	0.5

*Coefficients for specific growth rate (K) in the modified Ratkowsky model (Eq. 5).
**Coefficients for the inverse of lag phase duration (1/λ) in modified Ratkowsky model (Eq. 5).

experimental values indicates that the Gompertz model can accurately estimate the initial and final cell concentrations. In general, the Gompertz model can accurately describe the growth of *C. perfringens* in cooked beef under isothermal conditions from the lag, through the exponential, and to the stationary phases.

Effect of Temperature on Growth Parameters

The effect of temperature on specific growth rate and lag phase duration of *C. perfringens* was successfully analyzed using the modified Ratkowsky model (Table 1). The pseudo-R^2 values for K and $1/\lambda$ of the modified Ratkowsky models were 0.937 and 0.974, respectively. In Table 1, the standard errors of a and T_{max} are much smaller than those of b and T_{min}. This observation is not uncommon for the Ratkowsky models. In the study reported by Zwietering *et al.* (1991), the standard errors of a and T_{max} were also much smaller than those of b and T_{min} for two different forms of Ratkowsky models evaluated by the authors.

Depending on the products, the reported minimum, optimum, and maximum growth temperatures for *C. perfringens* are around 10-15C, 43-47C, and 50-52C, respectively (Labbe 1989; Wrigley 1994; FDA 1998). In this study, the minimum, maximum, and maximum temperatures estimated from the modified Ratkowsky model were 9.8, 47.1, and 50.8C, respectively. Therefore, the theoretical minimum, optimum, and maximum growth temperatures for C. *perfringens* in cooked beef observed in this study agreed closely with these values reported in the literature.

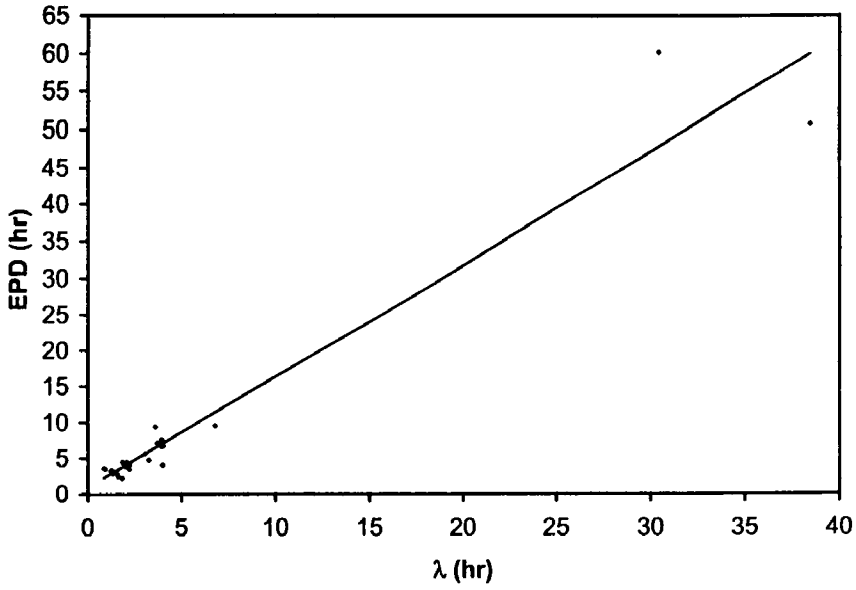

FIG. 2. LINEAR RELATIONSHIPS BETWEEN THE DURATIONS OF THE LAG AND EXPONENTIAL PHASES

In food microbiology, bacterial growth is typically categorized by a three-phase process - lag, exponential, and stationary in a linear model. The lag phase duration and specific growth rate under an isothermal condition can be readily derived from the primary Gompertz model. The duration of the exponential phase, however, has not been well investigated. Mathematically, the duration of an exponential phase (EPD) of an isothermal growth process can easily be derived from the four-parameter Gompertz model (Eq. 5). With this definition, a linear relationship (Fig. 2) was observed between the durations of the lag and exponential phases (Eq. 6, $R^2 = 0.943$).

$$EPD = \frac{B - A}{K} \tag{5}$$

$$EPD = 0.980 = 1.5333\ \lambda \tag{6}$$

The observation of the linear relationship between the durations of the lag and exponential phases may be of practical application. In most published studies concerning predictive microbiology, only the lag phase duration and specific growth

rate are reported. However, a complete growth curve cannot be established from these two parameters since the stationary phase cannot be determined. With the linear relationship between the lag and exponential growth phases (Eq. 6), the initial cell concentration, lag phase duration and specific growth rate can be used to generate complete linear growth curves containing the lag, exponential, and stationary phases without complicated mathematical computation (Eq. 7).

$$\begin{aligned} &L = A, \text{ at } 0 \le t < \lambda \\ &L = A + K(t - \lambda), \text{ at } \lambda \le t \le EPD + \lambda \\ &L = B, \text{ at } t > EPD = \lambda \end{aligned} \tag{7}$$

Comparison with Broth Data

Juneja *et al.* (1999) investigated the growth of *C. perfringens* in a broth system using a mixture of bacterial strains identical to this study. The specific growth rates of this organism determined from ground beef in this study were generally higher than those reported by Juneja *et al.* (1999), particularly at high temperatures (Fig. 3).

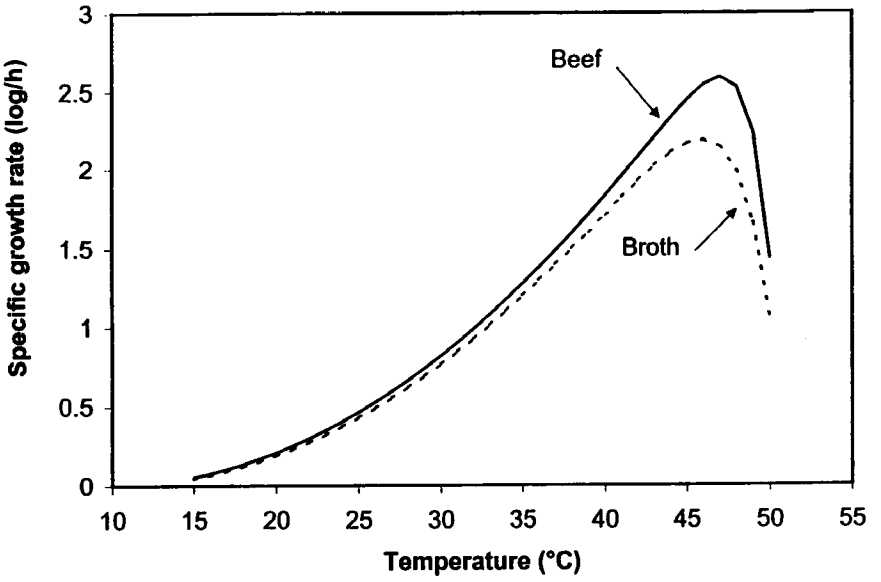

FIG. 3. COMPARISON OF SPECIFIC GROWTH RATES ESTIMATED FROM THE MODIFIED RATKOWSKY MODELS

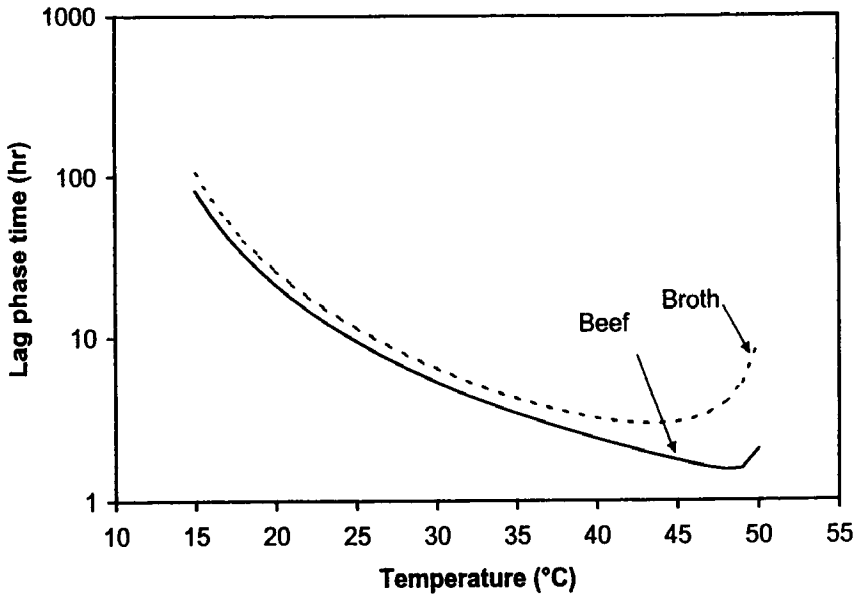

FIG. 4. COMPARISON OF THE LAG PHASE DURATIONS ESTIMATED FROM THE MODIFIED RATKOWSKY MODELS

The lag phase durations of this organism in the broth system were generally longer than those in ground beef (Fig. 4). The longer lag phases and smaller growth rates for the vegetative cells of *C. perfringens* in the broth system can be explained by the fact that the tryticase-peptone-glucose-yeast (TPGY) extract broth used by Juneja *et al.* (1999) may lack some nutrients critical to the bacterial cells. Similar observations were reported in the literature. In studies conducted by Willardsen *et al.* (1978; 1979), *C. perfringens* was found growing faster in autoclaved ground beef with shorter lag phases than in fluid thioglycollate medium (FTG). Willardsen *et al.* (1979) suggested that nutritional inadequacies or the presence of sodium thioglycollate in FTG may have prevented optimal growth of *C. perfringens*. The kinetic data reported in the present study suggested that using the kinetic models determined from the broth system potentially may lead to underestimation of bacterial growth in cooked beef.

CONCLUSION

This study investigated the growth kinetics of *C. perfringens* in cooked beef under isothermal conditions and used a four-parameter Gompertz equation as a primary model to describe the bacterial growth. This study also developed a secondary kinetic model to describe the temperature-dependence of the growth of *C. perfringens* in cooked beef.

The analysis of the experimental data revealed that the theoretical minimum, optimum, and maximum growth temperatures for *C. perfringens* in cooked beef were 9.8, 47.1, and 50.8C, respectively. The specific growth rates of *C. perfringens* in cooked beef were found higher to the data collected from a broth system (TPGY broth extract) using the same cocktail of the organism (Juneja *et al.* 1999), while the lag phase durations were shorter in cooked beef when compared with the same study.

This study also observed a linear relationship between the durations of the lag and exponential phases of the growth curves. This linear relationship can be potentially used in categorizing bacterial growth. With the specific growth rate and the linear relationship between the durations of the lag and exponential phases, a complete isothermal linear growth curve containing the lag, exponential, and stationary phases can be generated.

The kinetic models and growth parameters developed in this study can be potentially applied to the food industry to evaluate the growth of *C. perfringens* in cooked beef products under temperature abuse conditions. These models may provide a guide for designing appropriate cooling procedures for thermally processed beef products and avoid the outgrowth and multiplication of *C. perfringens* in finished products. The kinetic models also can be used to estimate the growth of *C. perfringens* in cooked beef products during storage and distribution.

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